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Preparation of cells for immunofluorocytometric analysis of cell surface markers (FACS)

Stock solutions to have on hand:

- 10x PBS, pH 7.4
- 20% NaN_3
- 500mM EDTA, pH 8.0
- Formaldehyde (37.5%)

Solutions you will use:

- PBS
- PBS + 2mM EDTA
- PBA (PBS + 1% BSA + 0.1% NaN_3)
- PBA + 1% formaldehyde

Note on cell number: The number of cells you need will depend on the type of experiment. In general, you want to count 10,000 cells (10^4). If you are looking at an endogenous marker and most of your cells are alive, in theory you wouldn't need to harvest more than about double that amount to get enough cells. In practice, you need at least 2 x 10^5 cells to obtain a large enough pellet to work with. You may need more if you are looking at a transfected marker. Another consideration is the time it takes to measure each sample. The more cells you have, the faster it will go. If you can get 10^6 cells or more you will spend less time sitting at the FACS watching cells go by.

Notes on experimental design: You always need at least one sample that is unlabeled. You can use an isotype control antibody, no primary antibody, or untransfected cells where appropriate. If you are measuring a transiently transfected marker you will usually need an independent transfection marker as well. The easiest way is to cotransfect eGFP or eYFP. Alternatively you can express your protein of interest as a fusion protein with eGFP or eYFP, or you can cotransfect another surface marker that you can detect with antibodies (common choices are CD4, CD8 and CD25 (Tac) because they are easy to express and fluorescent antibodies for these markers are inexpensive). For two color experiments, you will want singly labeled samples as controls. This is most important for fluorophore pairs with significant spectral overlap (e.g. FITC and PE). This is not the place to discuss this, but you will need these samples to do compensation when required.

Harvest cells (on bench top at RT except where noted):

Suspension cells: Harvest cells by centrifugation. If you have a large volume use a clinical centrifuge, 500 - 1000 x g, 5 min. Otherwise, spin down the cells in Eppendorf tubes. Use a "short" spin, allowing the rotor to get to top speed and then stop. This should take less than 20s. Aspirate the media and resuspend the pellet in 1 ml PBS (at this point you can transfer everything to Eppendorfs and use "short" spins for all subsequent steps).

Adherent cells: Aspirate media from plates. Wash by gently adding PBS (choose a volume based on plate size, e.g. 5 ml for 10 cm, 1 ml for 6-well (35 mm)). Aspirate and add a small volume of PBS + 2mM EDTA (2.5 ml for 10 cm, 500 μl for 6-well). Put plates in a 37°C incubator for 5 min. Pipette up and down to release cells and transfer to Eppendorfs. Very tightly adherent cells may require scraping, but if they don't disaggregate they're not a cell type you want to use for FACS! Spin down pellet, aspirate supernatant and resuspend in 1 ml PBS.

Incubate with primary antibody (on ice):

Spin down the cells and resuspend in 50 - 100 μl ice cold PBA + primary antibody. Antibody dilutions typically range from 1:50 to 1:200 and must be determined empirically by titration. Resuspension volume depends on the size of the pellet. Once you have introduced fluorophores (either this step or the next) you should keep your tubes away from bright light. Aluminum foil over your ice bucket is the easiest way. 30 min - 1 h.

Incubate with secondary antibody, if necessary (on ice):

Spin down cells. Wash pellet 2 or 3 times by resuspension in 1 ml ice cold PBS. Usually a simple vortex rather than pipetting up and down will suffice and complete resuspension is unnecessary. After washing, spin the cells down and resuspend (for real this time) in 50 - 100 μl ice cold PBA + secondary antibody. A 1:100 dilution is a good rule of thumb. 30 min - 1 h.

Notes on antibodies and fluorophores: The simplest situation is when you have monoclonal antibodies directly conjugated to fluorophores. These are widely available for most immunological markers and are fairly cheap. Alternatively you can make your own. Otherwise you must use primaries from different species (i.e. rabbit and mouse) and the appropriate fluorophore- conjugated secondaries. Choice of fluorophores will depend on the setup of your FACS, but are most typically FITC (a better alternative is AlexaFluor 488), PE, and APC. For two color experiments, AlexaFluor 488 and APC are my preferred combination because of the complete lack of spectral overlap and the brightness of the fluorophores.

Fixation:

Spin down cells. Wash pellet 2 or 3 times with 1 ml ice cold PBS. After washing, spin the cells down and resuspend in 100 μl PBA + 1% formaldehyde. The cells can be stored for up to one week at 4°C in the dark.

Preparation of samples for FACS:

The cells need to be diluted in PBS and filtered before they can be put into the FACS. The simplest way is to use tubes with cell strainer caps (Fisher Scientific catalog #08-771-23). Pop off the caps and add 500 µl PBS (RT) to each tube. Recap. One at a time, invert each tube to wet the inner surface of the filter and pipet the fixed cell suspension into the well of the cap. It works best if the filter is half-wet and you pipet the cells directly over the hanging drop of PBS. Don't puncture the filter. Your cells should fall right into the tube. For those that don't (it's a crap shoot), a very gentle spin will do the trick. Pop the caps back off and your samples are ready for FACSing.